Understanding stress-induced immunosuppression: Exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes[1]

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ABSTRACT At present, the poultry meat and egg industry has gained a lot of ground, being viewed as a provider of a healthy alternative to red meat and other protein sources. If this trend is to be maintained, solutions must be found to improve resistance of chickens to disease, which often is weakened by stressful conditions. In poultry, stress-induced immunosuppression is manifested by failures in vaccination and increased morbidity and mortality of flocks. Currently, several modern cellular and molecular approaches are being used to explore the status of the immune system during stress and disease. It is likely that these new techniques will lead to the development of new strategies for preventing and controlling immunosuppression in poultry. Using quantitative reverse transcription-PCR assays, a broad spectrum of cytokine, chemokine, and their receptor genes can be quantified in birds and then be used as markers to assess the effects of stress on the immune system. Currently, we are investigating immune and endocrine interactions in the chicken, in particular the cells and molecules that are known to be involved in such interactions in mammals. We have evaluated the effects of corticosterone administration in drinking water on peripheral lymphocyte and heterophil cytokine and chemokine gene profiles. In particular, there seems to be effects on cytokine and chemokine mRNA expression levels in both lymphocytes and heterophils, especially expression of the proinflammatory cytokines interleukin (IL)-1β, IL-6, and IL-18 and chemokines C-C motif, ligand 1 inflammatory (CCLi1); C-C motif, ligand 2 inflammatory (CCLi2); C-C motif, ligand 5 (CCL5); C-C motif, ligand 16 (CCL16); C-X-C motif ligand 1 inflammatory (CXCLi1); and C-X-C motif ligand 2 inflammatory (CXCLi2), which are initially upregulated and are potentially involved in modulating the adaptive immune response. A chronic treatment with corticosterone downregulates proinflammatory cytokines and chemokines, suggesting that the delayed effects of chronic stress can suppress the immune response. Messenger RNA expression levels of transforming growth factor-β4 (TGF-β4) are also upregulated in cortisosterone-treated birds. It appears that the balance between T-helper (Th) 1 and Th2/T regulatory cytokine production is altered in conditions associated with significant changes in plasma corticosterone concentration. Experiments are underway to decipher the cytokine and chemokine responses to vaccination and bacterial challenge on the background of stress-induced immunosuppression.

Key words: stress, immune suppression, leukocyte, cytokine and chemokine response, chicken

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INTRODUCTION

Stress (often used to mean the stressor or the stress response) is an event that the body experiences in response to threatening conditions, which can be extreme and acute or less acute and chronic over an extended period of time. A stressor is any factor that elicits this response. The stress response is a very complex and multifaceted mechanism; it involves a series of behavioral, physiological, metabolic, and immunological reactions that the body uses to redistribute the demands placed on it, adapt to them, and survive. The concept of stress, first introduced by Selye (1936), has been constantly revised and developed by other scientists, who have considered stress a mechanism that does not just simply maintain homeostasis. The concepts of allosta-

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sis (coping with stress and achieving stability through change) introduced by Sterling and Eyer (1988) and allostatic load-overload (stressed out and failing to cope with changes) were introduced and applied to the operation of the immune system by McEwen and Stellar (1993) and have been useful in understanding how the body can cope efficiently or fail to adapt to events in daily life over time (McEwen and Seeman, 1999). Allostasis and allostatic load are very important concepts that can be applied to production animals such as chickens, which use the resulting adaptive responses not only for their own benefit but also to maintain physiological activities such as egg production and body growth.

In mammals, the integrated response to stress is made up of several neuroendocrine molecules of both the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, and these include catecholamines (noradrenaline and adrenaline) produced by the sympathetic nervous system and adrenal medulla, corticotropin-releasing factor, and vasopressin, and the secretion of adrenocorticotropin from the pituitary gland, leading to secretion of glucocorticoids (**GC**) by the adrenal gland (cortisol-corticosterone). Leukocytes, such as T, B, natural killer, and antigen-presenting cells, such as dendritic cells, carry receptors for stress hormones produced by the adrenal and pituitary glands. These hormones can therefore modulate the activities of immune cells, in particular the production of proinflammatory cytokines and chemokines. The cytokines and chemokines themselves can in turn modulate the activity of the hypothalamus and thus alter hormone production (Besedovsky and del Rey, 2000, 2006).

In the chicken, these interactions work slightly differently. Some differences can be explained by the different architectures of the mammalian and avian immune system. In terms of immune system, birds lack lymph nodes, and in fact the actual site of antigen presentation in avian species is not well understood (Kaiser et al., 2009). The influence of sympathetic nerve innervations may also be less important in birds. Birds have a different repertoire of proinflammatory cytokines and chemokines compared with mammals and tumor necrosis factor-α (TNF-α), one of the key proinflammatory cytokines, has not been found in birds (Kaiser et al., 2005).

In this paper, we review the current knowledge on stress-induced immunosuppression in the chicken and reflect on some immune measures that can be used to detect reduced immunity in stressed birds. In general, discussions in this review are limited to immunosuppressive aspects of noninfectious stressors; disease agents causing immunosuppression are not considered. Much of the information discussed here is derived from literature on birds (mainly chickens) and mammals (humans and rodents). Finally, we review some initial data on the evaluation of cytokine and chemokine gene profiles in a corticosterone-induced stress model and discuss their contribution to the stimulation or inhibition, or both, of the immune response in treated birds.

Stress-Induced Changes in Immune Response: Enhancing Versus Suppressing Effects

Stress has been widely studied in mammals and avian species, including chickens. Several investigations have shown that stress influences-modulates the immune system (i.e., it suppresses immune function under some conditions while enhancing it under others). However, information on the effect of stress on immune responsiveness on the background of proven clinical interventions is limited. Evidence thus far supports the hypothesis that the up- or downregulation of cellular and molecular components of the immune response is associated with stress-induced changes in host resistance to disease. Therefore, it is noteworthy that stress has been associated with reports of both increased and decreased resistance to infectious diseases, as well as other immune-mediated disorders, in humans and animals.

In chickens, stress status is associated with increased plasma corticosterone concentrations because corticosterone is the end product of the hypothalamic-pituitary-adrenal axis. It has also been established that corticosterone is responsible for many quantitative and qualitative changes in immune function. The most reported effect of stress on the immune system is the suppression of immune organs and immune cells (Davison and Flack, 1981; Davison et al., 1983; Gross and Siegel, 1983; Siegel, 1985; McFarlane and Curtis, 1989; Maxwell et al., 1992; Puvadolpirod and Thaxton, 2000; Post et al., 2003; Shini et al., 2004; Mumma et al., 2006; Shini et al., 2008a). A growing biomedical literature, however, indicates that stress may not be obligatorily immunosuppressive; the exposure of birds to stress, particularly acute stress, may also cause enhancement of immune functions.

Stress-Induced Enhancement of Immune Response. When discussing stress-induced response, it is important to appreciate that just as stress stimulates other systems of the body (e.g., metabolic and cardiovascular systems) and prepares the organism for the "fight or flight" response, it may enhance immune response and prepare the immune system subsequent challenges (Dhabhar, 2009). In humans and rodents, in contrast to chronic stress, acute stress significantly enhances antigen-specific cell-mediated immunity by inducing circulating leukocytes to marginate within the vasculature of organs, such as the skin (Dhabhar and McEwen, 1996, 1997; Dhabhar, 2000, 2002).

In chickens, a certain degree of stress is essential for maintaining normal biological functions (Zulkifli and Siegel, 1995). Prior stressful experiences, particularly during the neonatal stage (i.e., fasting early in life), have shown long-term benefits in helping chickens to better withstand high ambient temperatures as juveniles, compared with those fed ad libitum throughout the neonatal age (Zulkifli et al., 1994). It has also been previously demonstrated that stress may have benefi-

cial influences on diseases of parasitic origin (Gross, 1976, 1984). Moreover, resistance to *Escherichia coli* and *Staphylococcus aureus* in chickens is increased by behavioral stress (Gross and Colmano, 1969). In contrast, chickens housed under relatively low stress environments were more susceptible to *E. coli* (Siegel and Gross, 1965; Gross and Colmano, 1967), *S. aureus* (Larson et al., 1985), and some external parasites (Hall and Gross, 1975) than those housed under relatively more stressful environments.

As in other animals, in chickens, the immediate response to a stressor is to mobilize and produce glucose to meet the increased energy requirement, a process mediated by catecholamines (Assenmacher, 1973). This general stimulation also may enhance the formation of antibodies (Braun et al., 1971; Siegel, 1985). In our experiments with 7-wk-old chickens, we employed a chronic corticosterone model and demonstrated that 1 h after treatment with corticosterone in the drinking water, antibody responsiveness to infectious bronchitis virus (**IBV**) vaccine was increased (Figure 1); thereafter, IBV titers decreased significantly. It was thought that acute stress (i.e., initial corticosterone administration) induced the humoral-mediated immune response.

Several researchers have proposed diverse strategies to modulate immune function, including psychological and behavioral interventions that could produce positive endocrine and immune change (Kiecolt-Glaser et al., 1985, 1992; Huff et al., 2003; Andersen et al., 2004). Although it is not yet clear to what extent these positive immunological changes translate into an improvement of pathological aspects of diseases, the preliminary evidence seems to be promising (Glaser and Kiecolt-Glaser, 2005). Moreover, a stress-induced enhancement of immune function is likely to be beneficial in promoting immunity during wound healing (Viswanathan and Dhabhar, 2005), bacterial and viral infections (Deak et al., 1999; Edwards et al., 2006), and vaccination (Dhabhar and Viswanathan, 2005; Edwards et al., 2008). Paradoxically, endogenous and exogenous GC (corticosterone) are known to be powerful antiinflammatory agents and are useful as antiinflammatory drugs in inflammatory, autoimmune, and allergic diseases (Boumpas et al., 1993). They reduce the production of numerous mediators of inflammation, including proinflammatory cytokines, prostaglandins, and reactive oxygen and nitrogen species (Tomchek et al., 1991; Flaster et al., 2007). Glucocorticoids have beneficial effects against bacterial disease in which the major pathology involved local or generalized inflammation (Rhen and Cidlowski, 2005). In general, stress-induced increased levels of corticosterone play an important role in suppressing innate and cellular immune responses (Sapolsky et al., 2000).

Stress-Induced Suppression of Immune Response. There is enough evidence both in mammalian and avian literature that chronic activation of the stress system can have damaging effects on a wide range of organs, including the immune system. From experiments with poultry, it has been observed that immunosuppression has been associated with involution of immune organs (e.g., bursa of Fabricius, thymus, and spleen), lowered immunity, and increased morbidity (Siegel, 1971; Thaxton et al., 1974; Pilo et al., 1985; Freeman, 1987; Puvadolpirod and Thaxton, 2000; Post et al., 2003; Shini, 2004; Huff et al., 2005, 2006; Lin et al., 2006; Virden et al., 2007; Shini et al., 2008a,b). Particularly high concentrations of GC have immunesuppressive effects by inhibiting, for example, antibody

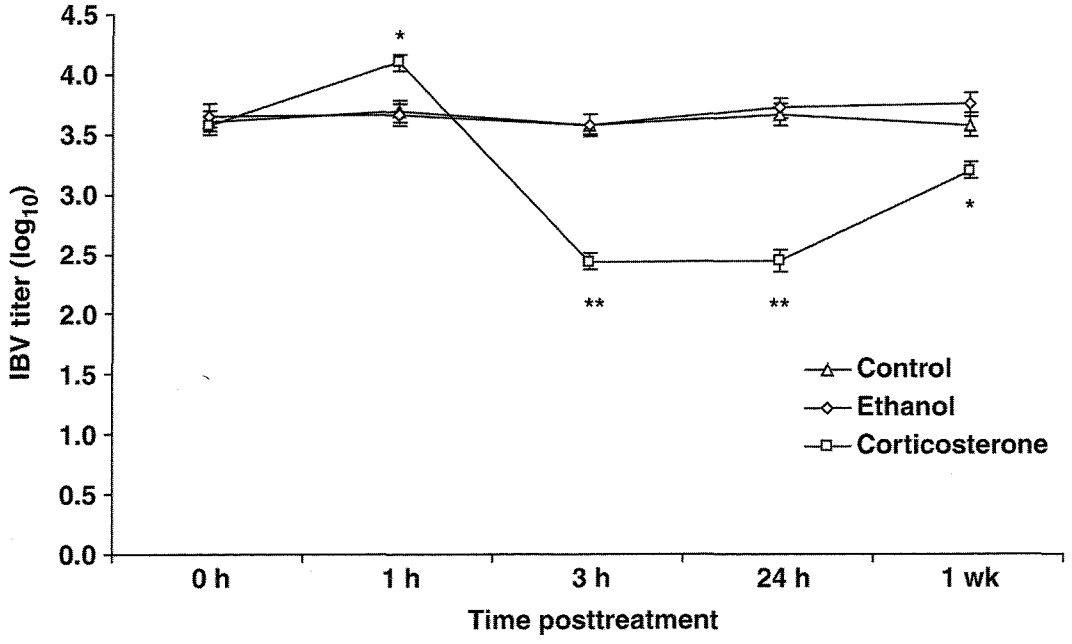

Figure 1. Antibody titers (log_{10}) to infectious bronchitis virus (IBV) vaccination of birds subjected to oral corticosterone and nontreated control birds. Values represent mean ± SEM, n = 12. Control = nontreated control chickens; ethanol = ethanol-treated control chickens; corticosterone = corticosterone-treated chickens. Asterisks denote significant differences between groups at each time point (*$P < 0.05$; **$P < 0.01$).

production from B cells and T-cell proliferation and phagocytosis (Davison et al., 1988; Bateman et al., 1989; Engler and Stefanski, 2003).

The immunosuppressive effects of GC on immune cells are thought to be mediated primarily through the cytoplasmic GC receptor (Bamberger et al., 1996) and its migration to the nucleus (Buttgereit and Scheffold, 2002). Glucocorticoid receptor elements can also be found in the promoter regions of numerous genes, and binding of GC receptors to these sites can alter gene expression (i.e., either express or suppress transcription) (Almawi and Melemedjian, 2002). However, it should be noted that there are several other mechanisms whereby GC can alter gene expression in immune cells and suppress immune functions.

Implications for the Poultry Industry. The chicken is one of the most domesticated animals and possibly also one of the animals longest maintained in captivity, continually subjected to some type of stress (Halverson, 2001). In the Gordon Memorial Lecture, Siegel (1995) commented on Selye's definition of stress and pointed out that for commercial birds, "the environment is a composite of interacting stressors that includes all the conditions in which they live—external (temperature, light, social or behavioural environments), as well as internal (disease organisms, toxins); therefore, the bird's success in coping with its environment depends on the severity of the stressors and its physiological ability to respond properly and to maintain or re-establish a homeostatic state." In poultry environments, stressors are present as a variety of physical, physiological, and infectious factors that may act alone or together and can affect the immune system by addition or synergy (Dohms and Metz, 1991; Dietert et al., 1994). Conditions such as crowding, handling, transport, food and water deprivation, exposure to unusual pathogens, unsanitary conditions, and malnutrition may cause prolonged and frequent high levels of plasma corticosterone, leading to a general reduction in immune competence and increased susceptibility to disease.

With regards to immunosuppression, the implications for the poultry industry are of major interest. Vaccinology is still a valid approach to producing specific immune responses effective in the prevention of diseases; however, vaccines are not available for most of the avian bacterial and parasitic infections. On the other hand, immunosuppression remains a hidden threat that could lead to vaccine failure and disease outbreak. Currently, stress costs the poultry industry in increased mortality and morbidity and in productivity losses. There are no research-based industry strategies for controlling stress and its biological cost in poultry. New biotechnological methods, such as early age administration of cytokines (as immunostimulatory adjuvants) or vaccines, or both, have only just started to demonstrate an increase in viability and disease resistance of chickens (Schat and Kaiser, 1997; Muir et al., 2000; Asif et al., 2004). On the other hand, modifying and improving the environment of the bird, in other words, minimizing the frequency or severity of the stressor(s), might increase production, reduce indices of disease, and enhance the quality of life of birds (Mench, 1992; Hester et al., 1996a,b). In intensive production systems, this might not be feasible because of significant economic and practical constraints. However, the use of environmental enrichment to meet the perceived needs of animals in captivity has increased accordingly. The benefits of an enriched cage system for laying hens range from changes in behavior (decreasing of aggression) to improved production and health (Tauson, 1986, 1998; Duncan, 1992; Appleby et al., 1993). It should be noted that in many cases, disease problems associated with intensive production systems are often husbandry problems. Therefore, fundamental improvements in husbandry practice, such as adequate conditions and supplies of food, fresh water, natural housing material, and construction, will give hens the space needed for movement and will reduce aggressive acts (i.e., stress).

Finally, in poultry production systems, as for other animal production systems, eliminating all stressors is impossible. One solution is to improve the ability of the bird to cope with production practices through genetic selection (e.g., developing selected breeding lines of laying hens that display far less aggression than their commercial counterparts) while maintaining industry-standard egg production and physiological status (Muir, 1996; Cheng et al., 2001; Cheng and Jefferson, 2008). Improving the response of an animal to stress through genetic selection for strains with lower stress response capacity and certain behavioral and physiological responses has been successful in laying hens (Muir and Craig, 1998; Cheng and Jefferson, 2008). The ability of laying hens to adapt to cages or alternative housing systems, or both, by means of genetic selection is also well documented (Hester et al., 1996a,b; Muir and Craig, 1998).

Immunosuppressive Effects of Stress: Lessons Learned from Corticosterone-Treated Chickens

Changes in Leukocyte Numbers and Proportions. From studies in humans, rodents, and birds, it has been established that the immune system responds to stress (i.e., elevated levels of corticosterone) with an increase of circulatory nonlymphoid leukocytes (neutrophils or heterophils in birds) and decrease of circulatory lymphoid leukocytes (lymphocytes). As a consequence, changes in the ratio of neutrophils (or heterophils) to lymphocytes occur. This reaction represents an evolutionarily conserved adaptive response that might contribute to an enhancement of the immune surveillance (especially antibacterial surveillance) in the organs-tissues of the "battle stations" in which leukocytes traffic during stress (Mishler, 1977; Davison and Flack, 1981; Munck and Guyre, 1991; Dhabhar, 1998, 2002). Therefore, the ratio of neutrophil (or het-

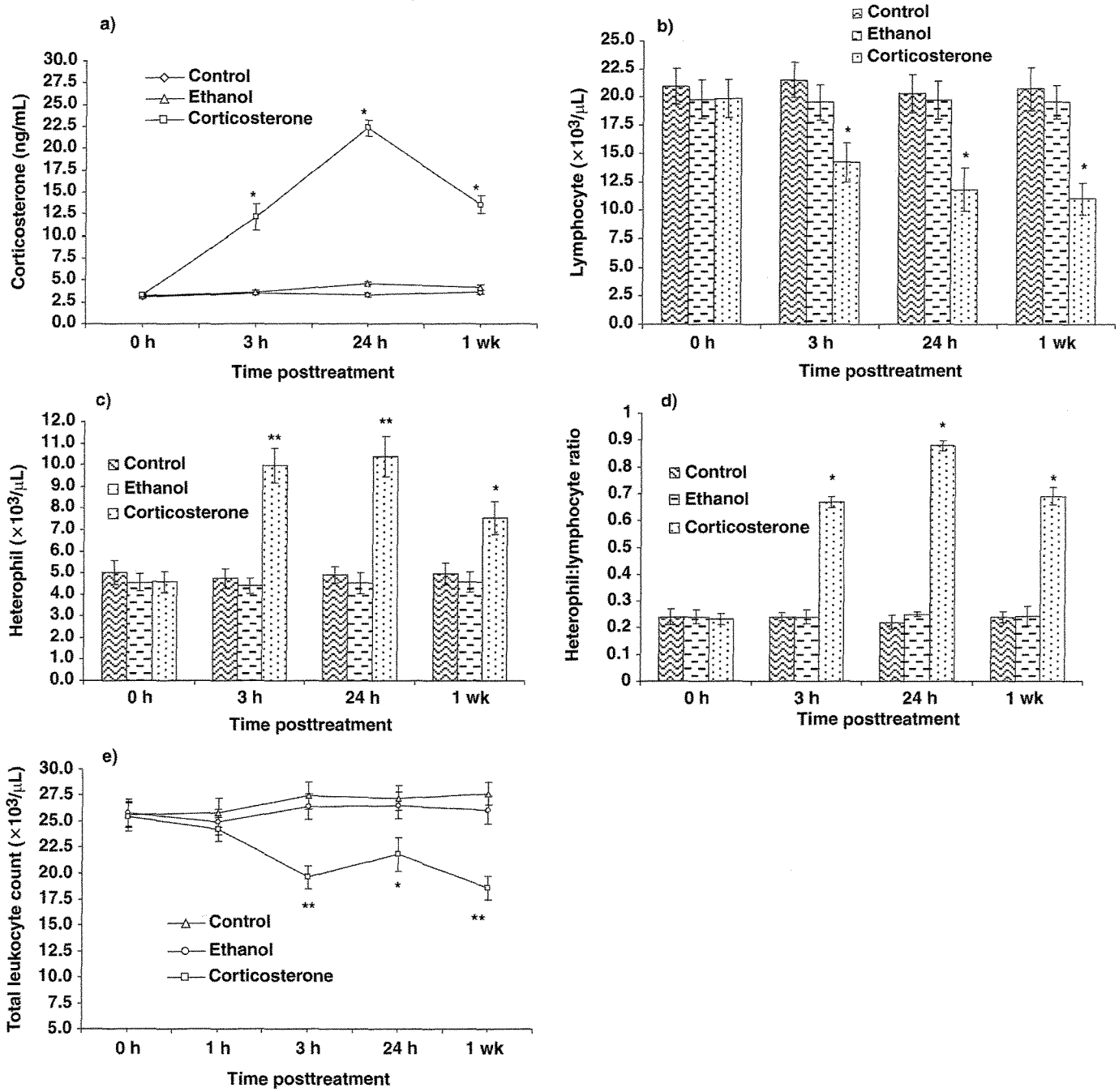

Figure 2. Effects of oral corticosterone administration on plasma corticosterone concentrations (a), lymphocyte counts (b), heterophil counts (c), heterophil:lymphocyte ratios (d), and total leukocyte counts (e) at 0 h (basal), 3 h, 24 h, and 10 d posttreatment with corticosterone. Values represent mean ± SEM, n = 12. Control = nontreated control chickens; ethanol = ethanol-treated control chickens; corticosterone = corticosterone-treated chickens. Asterisks denote significant differences between groups at each time point (*$P < 0.05$; **$P < 0.01$).

erophil) to lymphocyte has been used for years to assess stress in all vertebrates.

In our experiments with 7-wk-old chickens, we demonstrated that at 1 h, 3 h, and 24 h postadministration with exogenous corticosterone, plasma corticosterone concentration and heterophil:lymphocyte (**H:L**) ratios increased significantly (Figure 2a and d), and this was associated with a decreased peripheral lymphocyte count (Figure 2c) and increased peripheral heterophil count (Figure 2d), whereas the total circulating leukocyte number decreased (Figure 2e) (Shini, 2004, 2006; Shini et al., 2008a, 2009a,b). These observations confirmed that high circulating corticosterone concentrations may help leukocytes to redistribute among compartments, blood, lymphoid, and nonlymphoid tissue (Cohen, 1972), thereby bringing the cells required for the nonspecific response (i.e., heterophils) into the circulation. This could be a vital event for the successful development of the innate immune response within a stress response.

Changes in Immune Organ Weight as Related to BW. Spleen and bursa weight:BW ratio decreased after chronic treatment of birds with corticosterone in drinking water (Figure 3a). Compared with BW, the

inhibitory effect of corticosterone on the spleen and bursa weights was stronger; hence, a decrease in the relative weight of immune organs was observed 7 d post continuous treatments with corticosterone (Shini et al., 2008a). The suppression of lymphoid mass of the spleen was associated with a decrease in nonspecific phagocytic activity of splenic cells in immunosuppressed birds (Shini, 2004). Because chickens do not possess lymph nodes, it could be thought that the spleen (as a secondary lymphoid organ) might play an important role in immunological coordination and function. It has also been suggested that in adult chickens, mature T cells, B cells, and macrophages interact in the spleen during immunological responses (Grasman, 2002). Therefore, the mass and cellular activity of the spleen provide important information on immune status of birds and can be used to assess altered immunity and immunosuppression (Dohms and Saif, 1983; Dietert et al., 1994).

We also evaluated the swelling response, as quantified by cutaneous basophil hypersensitivity wattle reaction 24 h after the exposure to phytohaemagglutinin injections, in corticosterone-treated chickens. Treated birds demonstrated a reduced swelling response when compared with unexposed birds, suggesting suppression of the lymphoproliferative (primarily T-cell division) ability of the immune system (Grasman, 2002; Shini, 2004).

From our studies, it was demonstrated that elevations in plasma corticosterone concentrations induced retardation in lymphoid organs such as spleen and bursa of Fabricius. This, together with a decrease in circulating lymphocytes (T-cell response) and decreased antibody titers to IBV antigen (humoral-mediated response) (Figure 1), suggested that treated chickens experienced immunosuppression that started at least 3 h after treatment with corticosterone in drinking water.

In 1983, Dohms and Saif delineated immunosuppression and discussed the criteria for evaluating it in agricultural animals. They suggested that several immune approaches (morphometric, cellular, and humoral) may help to generate data that could provide evidence of immunosuppressive properties of a given agent. In their review, Dietert et al. (1994) discussed environmental factors that are known to modulate the immune system and influence performance and disease susceptibility. They also discussed and proposed potential immune measures that could be used to assess immune status in agricultural animal species (Dietert et al., 1994). In this paper, we have modified and updated this panel and recommend several biological markers of immune response that have shown to be successful in detecting stress-induced immunosuppression in poultry (Table 1). However, one should be reminded that the immune system may be influenced simultaneously by many factors (both infectious and noninfectious). Moreover, when assessing the effects of stress on immunity, it is important to exclude the presence of infectious diseases.

The Molecular Milieu Beyond H:L Ratios: Cytokine and Chemokine mRNA Expressions in Heterophils and Lymphocytes of Corticosterone-Treated Chickens. The H:L ratio has been used to assess stress for more than 3 decades; however, the molecular pathways beyond circulating heterophil and lymphocyte changes are poorly understood. Recently, the availability of the chicken genome sequence (International Chicken Genome Sequencing Consortium, 2004) has been responsible for much progress in deciphering the cellular and molecular mechanisms of the immune response. The precise content and order of the chicken genome, which is carried on 40 chromosomes, is almost complete. The assembled genome sequence provides a good basis to develop new markers that can be

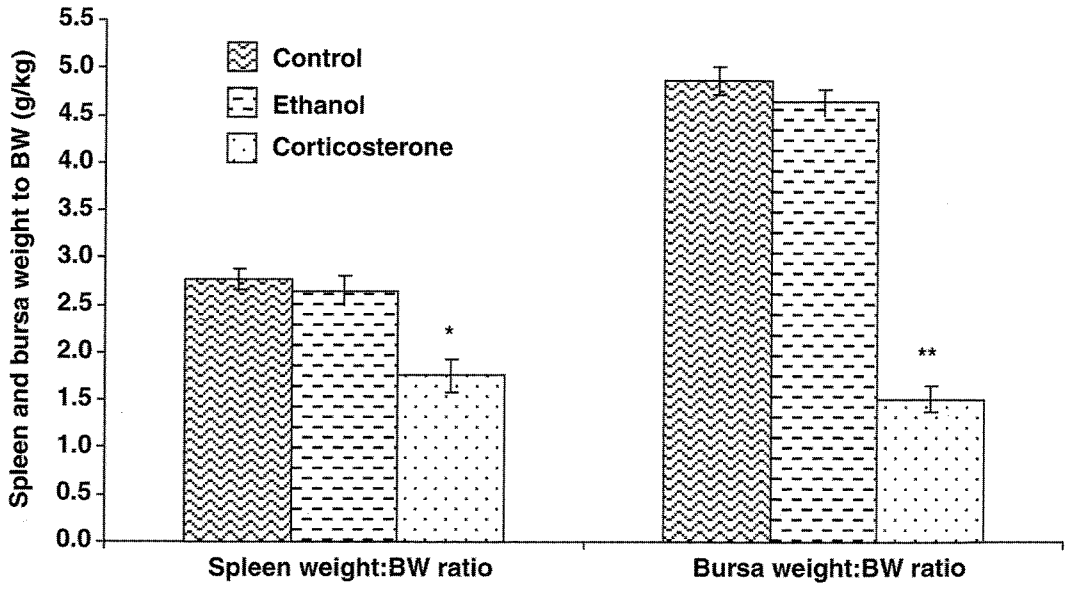

Figure 3. Spleen and bursa weights (g/kg of BW) in birds subjected to oral corticosterone 1 wk posttreatment. Values represent mean ± SEM, n = 6. Control = nontreated control chickens; ethanol = ethanol-treated control chickens; corticosterone = corticosterone-treated chickens. Asterisks denote significant differences between groups ($P < 0.05$).

Table 1. The immune test panel for poultry—an update[1]

Immune test/assay	Immune component tested
1. General	
Immune organs	Thymus, bursa, spleen
	Organ weight:BW ratio
	Histological evaluation
2. Enumerative	
Total and differential leukocytes	Peripheral blood
	The number and percentages of different kinds of leukocytes in peripheral blood
	Thymus, spleen
	T cell numbers and ratios (CD4+ and CD8+)
3. Physiological	
Total and specific proteins	Plasma or serum concentration of total proteins
	Albumin
	Globulin (α, β, γ)
	Fibrinogen
	Immunoglobulin classes
	Complement levels
4. Functional	
Innate immune response	Natural killer cell activity
	Neutrophil/heterophil phagocytic function
	Peritoneal macrophage recruitment and activation
Acquired immune response (cellular and humoral response)	
In vivo tests: delayed hypersensitivity (antigen-specific) or cutaneous basophil hypersensitivity skin test response to a mitogen (e.g., phytohemagglutinin)	T-cell-mediated response
Quantifications of antibody titer to antigens (SRBC, keyhole limpet hemocyanin, lipopolysaccharide) or commercial vaccines	Antibody-mediated response
In vitro tests: lymphocyte response to a mitogen (prolifereation/blastogenesis); ELISpot	T- and B-cell-mediated response
5. Immune molecules/genes	
Functional and antibody-based techniques	Plasma cytokine/chemokine levels
Gene expression techniques: real-time PCR or quantitative reverse transcription-PCR assays (the only way to detect many avian cytokines and chemokines)	Gene expression levels of cytokines/chemokines in immune cells from peripheral blood or immune organs

[1]The list of tests is an update of the panel recommended by Dietert et al. (1994). It is suggested that a combination of several tests might help to assess immunosuppression; specific immune assays from this panel might be more suitable for field or research studies, or both.

used in immunological studies. More than 23,000 genes have been identified so far, of which many have a role in immunity, including cytokine and chemokine genes.

It has now been demonstrated that, as in mammals (Agarwal and Marshall, 2000; Elenkov, 2004; Viswanathan and Dhabhar, 2005; Calcagni and Elenkov, 2006), stress in chickens can influence the immune response and alter cytokine and chemokine responses (Hangalapura et al., 2006; Shini and Kaiser, 2009). We have started to link changes in lymphocyte and heterophil number, distribution, and morphology with the levels of cytokine and chemokine mRNA expression in these cells. To date, our work focused on the effects of corticosterone on cytokine and chemokine gene expression profiles in heterophils and lymphocytes because of the critical role that these cells play in both immune and stress responses. We have examined whether acute and chronic oral administration of corticosterone causes differences in cytokine and chemokine mRNA expression levels in peripheral lymphocytes and heterophils and whether these differences are detectable by quantitative reverse transcription-PCR. The mRNA expression levels of interleukin (**IL**)-1β; IL-2; IL-4; IL-6; IL-10; IL-12α; IL-12β; IL-13; IL-13; IL-18; interferon-γ (IFN-γ), transforming growth factor-β4 (**TGF-β4**), C-C motif, ligand 1 inflammatory (CCLi1), C-C motif, ligand 2 inflammatory (**CCLi2**), C-C motif, ligand 5 (**CCL5**), C-C motif, ligand 16 (**CCL16**), C-X-C motif ligand 1 inflammatory (**CXCLi1**), C-X-C motif ligand 2 inflammatory (CXCLi2), CXC chemokine receptor 1 (CXCR1), and CXC chemokine receptor 4 (CXCR4) in peripheral heterophils and lymphocytes of treated and nontreated chickens were recently determined (Shini and Kaiser, 2009; Shini et al., 2009b).

Results have revealed that there are effects on cytokine and chemokine mRNA expression, particularly upregulation of proinflammatory chemokines in both lymphocytes and heterophils. Proinflammatory cytokines, including IL-1$\beta\alpha$, IL-6, and IL-18, were also upregulated in lymphocytes 3 h after first treatment with corticosterone (Figure 4a). Compared with the control, higher expression of mRNA for chemokines, particularly CCLi2, CCL5 (RANTES), CCL16, and CXCLi1, from lymphocytes was detected in corticosterone-treated birds 3 h post initial exposure (Figure 4b and c). The TGF-β4 and IL-18 mRNA were elevated 1 wk post initial treatment with corticosterone. There was a positive correlation between plasma corticosterone concentrations and CCL5 and CCL16 at 3 h post initial administration. At 1 wk post initial treatment, corticosterone levels correlated positively with CCL5 and negatively with IL-18. Furthermore, we demonstrated that exposure to corticosterone significantly upregulated mRNA expression levels for proinflammatory interleukins (particularly IL-1β, IL-6, and IL-18) in circulating heterophils of treated

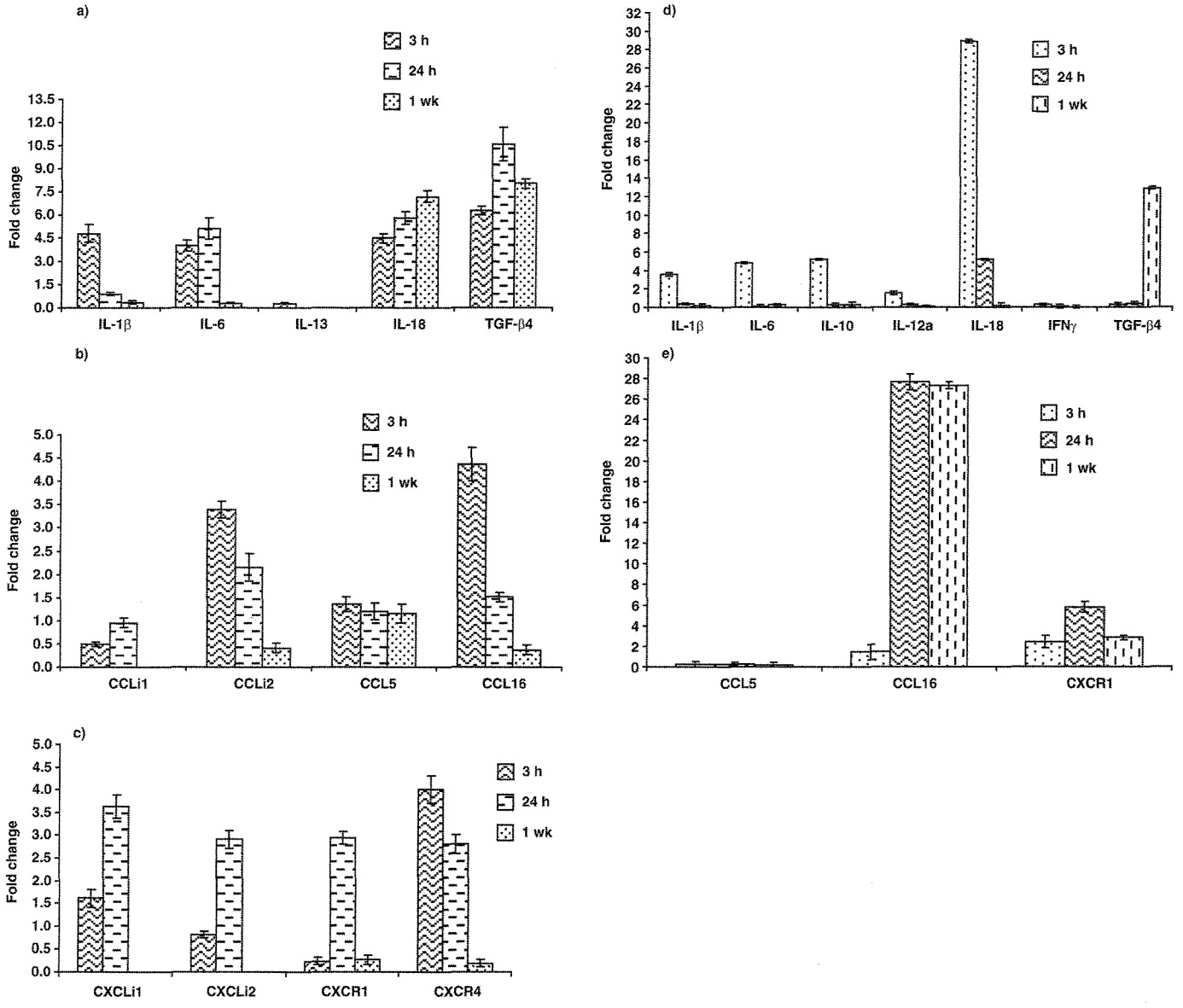

Figure 4. Fold change in cytokine (a) and chemokine (b, c) mRNA levels in peripheral lymphocytes and cytokine (d) and chemokine (e) mRNA levels in peripheral heterophils from corticosterone-treated chickens at 3 h, 24 h, and 1 wk after initial exposure, when compared with controls (untreated and ethanol-treated birds). Error bars show SEM from triplicate samples (n = 16) from 2 separate quantitative reverse transcription-PCR experiments ($P < 0.01$). IL = interleukin; TGF-β4 = transforming growth factor β4; IFNγ = interferon γ; CCLi1 = C-C motif, ligand 1 inflammatory; CCLi2 = C-C motif, ligand 2 inflammatory; CXCR1 = CXC chemokine receptor 1; CXCR4 = CXC chemokine receptor 4; CCL5 = C-C motif, ligand 5; CCL16 = C-C motif, ligand 16.

chickens. Although the initial treatment with corticosterone elevated the mRNA expression levels for many cytokines, such as IL-1β, IL-6, IL-10, IL-12α, and IL-18 (Figure 4d), only proinflammatory cytokines were downregulated at 3 and 24 h post corticosterone treatment. The mRNA expression levels for IL-6, IL-10, and IL-18 correlated with plasma corticosterone concentration and total heterophil counts. Repeated and chronic treatment with corticosterone in drinking water upregulated mRNA expression levels for TGF-β4 (Figure 4d) and the chemokine CCL16 (Figure 4e).

All data together indicate that cytokine and chemokine gene expression signatures in chicken lymphocytes and heterophils are altered during stress and therefore could be used as stress indicators in chickens. In these experiments, we were able to show that initial treatment with corticosterone not only altered the number of circulating lymphocytes and heterophils but also transcriptionally upregulated mRNA expression levels of proinflammatory-antiinflammatory cytokines and chemokines. These observations were the first demonstration of the inducible expression of proinflammatory molecules in avian immune cells by a stress hormone. Work is underway to explore the cytokine and chemokine profiles in both vaccinated or challenged and immunosuppressed birds, or both. Further research is needed to understand endocrine and immune molecule cross-talk in avian species and especially pathways by which innate and acquired cells are involved in the stress response.

The Regulation of the T-Helper 1, T-Helper 2, and T Regulatory Responses: Stress as a "Double Agent"? It is well known that to balance self-tolerance and protection against foreign agents, the immune system depends on both activation mechanisms and downregulatory mechanisms (Van Maren et al., 2008). Cytokines produced by T-helper (**Th**) 1, 2, and 17 and T regulatory (**Treg**) lymphocytes play a major role in this coordination. It is not surprising that initial treatment with corticosterone activated the expression of proinflammatory cytokines and chemokines (corticosterone is an antiinflammatory agent); simultaneously, TGF-β4 (primarily an antiinflammatory cytokine produced by Th2/Treg cells) was also upregulated. In contrast to the proinflammatory response, the later antiinflammatory response appears to protect the body from an "overshoot" with proinflammatory cytokines and chemokines. Transforming growth factor-β (**TGF-β**) is a regulatory cytokine with pleiotropic functions in T-cell development, homeostasis, and tolerance (Li et al., 2006). Recent studies with mice have indicated that Treg-derived TGF-β is required to control exaggerated Th1, Th2, and Th17 responses (Li et al., 2007). Our studies suggest a shift from predominantly Th1 cells to Th2/Treg cells. The Treg cells [Treg type 1 (**Tr1**) cells, Th3, and CD4+CD25+ T cells] also called "adaptive Tregs" are known to exert suppression via the secretion of soluble factors, such as antiinflammatory cytokines (Van Maren et al., 2008). Once activated, Treg cells are able to suppress T-cell proliferation and cytokine production, as well as antigen-presenting cell function. Control of Treg function is known to occur through cytokines such as IL-1, IL-6, and IL-12 and multiple costimulatory molecules expressed by antigen-presenting cells (Sutmuller et al., 2006). The Tr1 cells mediate suppression by secreting high amounts of IL-10; Th3 cells also secrete high amounts of TGF-β themselves. The information on the role of Treg cells in the control of immune responses in chickens is still limited; however, as in mammals, they seem to play an important role in the protection of cells by suppressing the harmful activation of the immune system.

Although stress begins in the brain, it also affects the brain as well as the rest of the body. Acute stress responses promote adaptation and survival via responses of neural, cardiovascular, metabolic, and immune systems. Conditions associated with acute significant changes in corticosterone levels might affect the immune system by increasing proinflammatory responses, leading to potential modulation of the Th1-Th2/Treg balance. A well-balanced Th1-Th2/Treg response would optimize the immune response. Obviously, chronic elevation of plasma corticosterone suppresses the immune response through the Treg system and TGF-β. From the evidence discussed here, it appears that "good stress" is linked to acute and moderate stress, whereas "bad stress" emerges when acute stress becomes repeated and chronic. The challenge would be to find the boundary and transcriptionally quantify "optimum stress."

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